

Leukotriene B₄ stimulation of phagocytes results in the formation of inositol 1,4,5-trisphosphate

A second messenger for Ca²⁺ mobilization

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Inositol trisphosphate (InsP₃) production and cytosolic free Ca²⁺ ([Ca²⁺]_i) elevations induced by leukotriene B₄ (LTB₄)-receptor activation were studied in the human promyelocytic-leukaemia cell line HL60, induced to differentiate by retinoic acid. The myeloid-differentiated HL60 cells respond to LTB₄ by raising their [Ca²⁺]_i with a dose–response relationship similar to that shown by normal human neutrophils. The observations of the LTB₄ transduction mechanism were compared with those of the transduction mechanism of the chemotactic peptide fMet-Leu-Phe in HL60 cells differentiated with dimethyl sulphoxide. Both LTB₄ and fMet-Leu-Phe triggered a rapid (< 5 s) elevation of [Ca²⁺]_i, which occurred in parallel with the InsP₃ production from *myo*-[³H]inositol-labelled cells. The threshold concentrations of the agonists, for InsP₃ production, were found at 10^{−9} M, a slightly higher concentration than that required to detect [Ca²⁺]_i elevations. No significant changes were noted in the phosphoinositide levels upon stimulation with LTB₄. Exposure to *Bordetella pertussis* toxin before LTB₄ stimulation abolished both the increased formation of InsP₃ and the rise of [Ca²⁺]_i. LTB₄ and fMet-Leu-Phe elicited elevations of inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] with no detectable lag time, followed by slower and more sustained inositol 1,3,4-trisphosphate elevations. Stimulation with various leukotriene analogues revealed a good correlation between both total InsP₃ as well as Ins(1,4,5)P₃ formation and elevations of [Ca²⁺]_i. Thus LTB₄ receptor activation results in an increased production of Ins(1,4,5)P₃ via a transduction mechanism also involving a nucleotide regulatory protein, as previously described for the fMet-Leu-Phe transduction mechanism.

INTRODUCTION

Leukotriene B₄ (LTB₄) has been shown to be a major arachidonic acid metabolite of the lipoxygenase pathway of neutrophils (Borgeat & Samuelsson, 1979). Furthermore, LTB₄ is a potent chemotactic factor that also induces adhesion, aggregation and secretion in human neutrophils (Ford-Hutchinson *et al.*, 1980; Palmblad *et al.*, 1981; Samuelsson, 1983). Consequently, LTB₄ most likely plays an important role as a potentiator of the inflammatory process in a wide variety of diseases. The described cellular responses of neutrophils are triggered by LTB₄ interacting with specific cell-surface receptors. In human neutrophils, receptors with both high and low affinities for LTB₄ have been demonstrated (Goldman & Goetzl, 1984). One of the earliest events after LTB₄-receptor interaction is a rise of the cytosolic free Ca²⁺ concentration, [Ca²⁺]_i, a critical signal in eliciting neutrophil responses (Lew *et al.*, 1984a). The fact that LTB₄ is able to raise [Ca²⁺]_i in the absence of extracellular Ca²⁺ concentrations reflects mobilization of Ca²⁺ from intracellular pools (Lew *et al.*, 1984a).

Considerable progress has been made in recent years

in the understanding of receptor-mediated mobilization of intracellular Ca²⁺. A wide variety of hormones and neurotransmitters stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] by phospholipase C (also referred to as phosphoinositide diesterase), resulting in the formation of two distinct intracellular second messengers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and diacylglycerol (for a review, see Berridge & Irvine, 1984). Ins(1,4,5)P₃ releases Ca²⁺ from an intracellular compartment, presumably the endoplasmic reticulum (Prentki *et al.*, 1984a). This process has been well documented in human neutrophils (Prentki *et al.*, 1984b), as well as in differentiated HL60 cells (Burgess *et al.*, 1984), a promyelocytic-leukaemia cell line. It has been shown that phagocyte activation by the chemotactic peptide fMet-Leu-Phe is mediated, at least in part, by enhanced phospholipase C hydrolysis of PtdIns(4,5)P₂ and increased formation of Ins(1,4,5)P₃ (Burgess *et al.*, 1985).

The question arises as to whether LTB₄ mobilizes intracellular Ca²⁺ by the same pathway. On the basis of results obtained by ³²P-labelling of phospholipids in rabbit neutrophils, it was concluded that this did not

Abbreviations used: LTB₄, Leukotriene B₄ [5(S),12(R)-dihydroxyeicosa-6,14-*cis*-8,10-*trans*-tetraenoic acid]; fMet-Leu-Phe, formylmethionyl-leucylphenylalanine; quin2, methoxyquinoline derivative of bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid; quin2/AM, tetra-acetomethyl ester of quin2; DMSO, dimethyl sulphoxide; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; InsP, inositol monophosphate; InsP₂, inositol bisphosphate; InsP₃, inositol trisphosphate; Ins(1,3,4)P₃, inositol 1,3,4-trisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; PMA, phorbol myristate acetate; [Ca²⁺]_i, cytosolic free Ca²⁺.

occur at chemotactic concentrations of LTB_4 (Volpi *et al.*, 1984). In contrast, Bradford & Rubin (1985a) reported a small, but significant, increase of InsP_3 production after stimulation of rabbit neutrophils with 10^{-7} M- LTB_4 . However, the latter observation does not resolve the controversy, since the concentration used was at least 100-fold higher than that necessary to mobilize intracellular Ca^{2+} and no attempts were made to characterize the nature of the InsP_3 produced. A qualitative and quantitative correlative analysis of total InsP_3 , $\text{Ins}(1,4,5)\text{P}_3$ and $[\text{Ca}^{2+}]_i$ elevations in retinoic acid-differentiated HL60 cells in response to LTB_4 and its analogues forms the basis of the present paper.

EXPERIMENTAL

Chemicals

The materials and their sources were as follows *Bordetella pertussis* toxin (List Biological Laboratories, Campbell, CA, U.S.A.); fMet-Leu-Phe, PMA, cytochalasin B and cytochrome *c* (Sigma Chemical Co., St. Louis, MO, U.S.A.); *myo*-[2- ^3H] inositol, quin2/AM and quin2 (Amersham International, Amersham, Bucks., U.K.); Dextran T500 and Ficoll-Hypaque (Pharmacia Fine Chemical, Uppsala, Sweden); LTB_4 (Calbiochem, San Diego, CA, U.S.A.); and 4-methylumbelliferyl substrates (Koch Laboratories, Haverhill, Suffolk, U.K.). Ionomycin was kindly given by C. M. Liu, Hoffmann-LaRoche, Nutley, NJ, U.S.A.

Culture of HL60 cells

The cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal-calf serum, penicillin (100 units/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). The cells were passaged once every week, and only cells with a passage number below 35 were used for the experiments. Differentiation of the cells was induced by adding DMSO (final concn. 1.3%, v/v) or retinoic acid (final concn. 0.2 μM) to the cell suspensions. All experiments were performed on cells differentiated for 4 days. The Nitro Blue Tetrazolium test (NBT test) was performed as previously described by stimulation of the cells with 100 nM-PMA (Zimmerli *et al.*, 1984).

Measurement of cytosolic free Ca^{2+}

Quin2 loading was performed as previously described (Lew *et al.*, 1984b). Cells, washed and suspended at a concentration of $5 \times 10^7/\text{ml}$, were equilibrated at 37 °C for 5 min in a medium containing 138 mM-NaCl, 6 mM-KCl, 1 mM- MgSO_4 , 1.1 mM- CaCl_2 , 0.1 mM-EGTA, 1 mM- Na_2HPO_4 , 5 mM- NaHCO_3 , 5.5 mM-glucose, and 20 mM-Hepes, pH 7.4. quin2/AM was added to final concentrations of 10, 15 or 20 μM from a 10 mM stock solution in DMSO. Control cells were incubated in equal amounts of DMSO. At 10 min after the addition of quin2/AM or DMSO, the cells were diluted to $1 \times 10^7/\text{ml}$ with warm medium containing 0.5% bovine serum albumin and left for another 50 min at 37 °C. After this loading the cells were kept at room temperature until used. Just before use, a sample of the cells was centrifuged and resuspended in the same type of medium. Fluorescent measurements were performed with a Perkin-Elmer fluorimeter (LS3; Perkin-Elmer Corp., Norwalk, CT, U.S.A.). The cells ($1.8 \times 10^7/\text{ml}$) were kept in suspension by magnetic stirring at 37 °C in a thermostatically controlled cuvette holder. Excitation and emission wave-

lengths were 339 ± 5 nm and 492 ± 10 nm respectively. To minimize light-scattering artefacts, two cut-off filters (UV D25 and UV 35), for excitation and emission respectively, were used. Calibration of the quin2 fluorescence to yield changes of quin2 saturation was performed as previously described (Lew *et al.*, 1984b). Because differentiation of HL60 cells to functionally mature cells occurs in a subpopulation of the cell suspension, which complicates the calculation of $[\text{Ca}^{2+}]_i$ (Pozzan *et al.*, 1982), results are expressed as a percentage increase of quin2 saturation.

Measurement of phosphoinositides and inositol phosphates

Phosphoinositides and inositol phosphates were labelled by incubating cells with *myo*-[2- ^3H]inositol (1 $\mu\text{Ci}/\text{ml}$) in RPMI-1640 medium devoid of inositol supplemented with 10% fetal-calf serum during the last 48 h of differentiation. The decreased concentration of inositol had no effect on cell proliferation and differentiation, nor did it affect the ability of LTB_4 or fMet-Leu-Phe to generate Ca^{2+} signals or of phorbol myristate acetate to generate superoxide anions. Extension of the incubation with *myo*-[2- ^3H]inositol for 3–7 days did not increase the labelling of [^3H]inositol incorporation into phosphoinositides. For the determination of stimulus-induced changes in phosphoinositides and inositol phosphates the prelabelled cells were washed twice at 37 °C for 10 min in RPMI medium lacking inositol. The cells were then resuspended in Ca^{2+} medium, warmed for 5 min at 37 °C, followed by addition of various stimuli. Control cells were treated in parallel with appropriate solutions. We have initially compared directly inositol phosphate levels in quin2-loaded and unloaded cells, without detecting any significant differences. The number of cells used for each incubation condition was 3×10^6 . Incubations were terminated at the indicated times by addition of 10% (v/v) trichloroacetic acid. The samples were then put on ice for 5–10 min and centrifuged. The phospholipids in the trichloroacetic acid precipitate were extracted with 5.5 ml of chloroform/methanol/12 M-HCl (800:400:3). These extracts were then washed three times with 1 ml of chloroform/methanol/0.6 M-HCl (3:48:47) and dried under a stream of air. Phosphoinositides were analysed by t.l.c. as described by Jolles *et al.* (1981). The supernatant was also washed three times, but with a 5-fold excess of diethyl ether. The washed extract was adjusted to pH 7.5 with Tris (0.2 M) and the inositol phosphates were separated by stepwise elution from small Dowex anion-exchange columns as previously described (Berridge *et al.*, 1983). The radioactivity of the different fractions was determined by liquid-scintillation counting with 67% (v/v) Aquasol. To resolve between the two isomers of InsP_3 , the ether-washed and neutralized trichloroacetic acid extracts were analysed by h.p.l.c., essentially as previously described (Burgess *et al.*, 1985; Irvine *et al.*, 1985). After addition of ATP (10 μM) and EDTA (1 mM), the trichloroacetic acid extracts (maximally 1 ml) were chromatographed on a Partisil SAX 10 anion-exchange column (packed by Technicol Ltd., Stockport, Cheshire, U.K.) at a flow rate of 1.6 ml/min. An initial 5 min washing period in water was followed by a linear gradient of ammonium formate (the pH being adjusted to 3.7 with H_3PO_4). The gradient reached a concentration of 1 M after 24 min, and this final concentration was then maintained for an additional

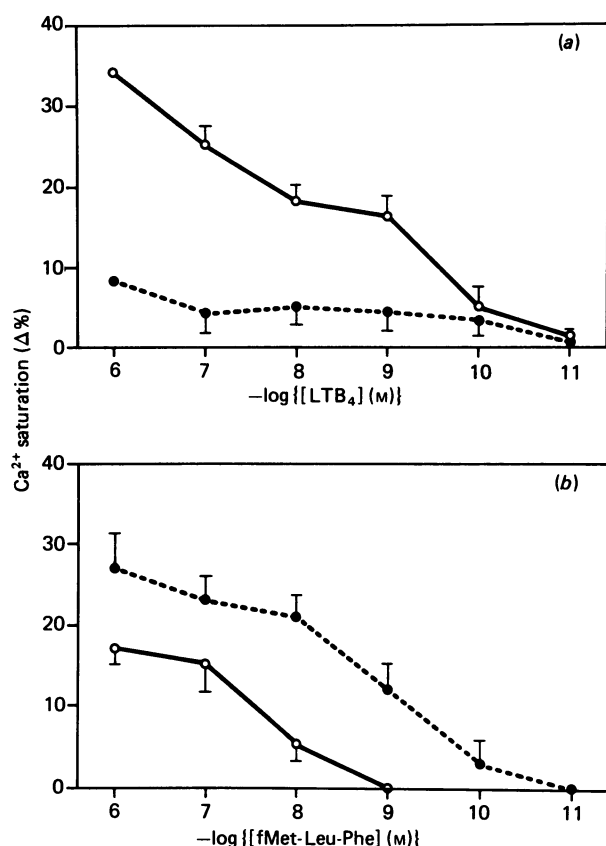


Fig. 1. LTB₄- and fMet-Leu-Phe-induced rises in $[Ca^{2+}]_i$

(a) Shows the dose response for LTB₄-induced $[Ca^{2+}]_i$ rises in both retinoic acid (○)- and DMSO (●)-differentiated HL60 cells. (b) Shows the dose response for fMet-Leu-Phe-induced $[Ca^{2+}]_i$ rises in both retinoic acid (○)- and DMSO (●)-differentiated HL60 cells. The changes in $[Ca^{2+}]_i$ were monitored with the fluorescent probe quin2. The intracellular concentration of quin2 was 0.32 ± 0.09 nmol/ 10^6 cells. The $[Ca^{2+}]_i$ values represent the peak reached in each trace and are the mean and S.D. for three separate experiments.

period of 5 min. Fractions were collected at 0.3 or 0.4 min intervals. The $[^{32}P]$ Ins (1,4,5) P_3 that served as a standard was generously provided by Dr. R. Irvine, ARC Institute of Animal Physiology, Babraham, Cambridge, U.K. Note that the designation of the Ins P_3 isomers in the present study is based on their chromatographic behaviour on h.p.l.c. and relies on the structural analysis performed previously by Irvine *et al.* (1984).

Measurement of superoxide production

Superoxide production by HL60 cells was monitored continuously in a double-beam spectrophotometer, thermostatically controlled at 37 °C as previously described (Lew *et al.*, 1984b).

Incubation with *Bordetella pertussis* toxin

A 100 μ g/ml stock solution of the toxin was made by diluting 50 μ g of it in 500 μ l of a buffer containing 0.1 M-sodium phosphate and 0.5 M-NaCl, pH 7.0. Cells were suspended in Ca^{2+} medium at a concentration of 10^7 /ml and incubated for 2 h at 37 °C with 100 ng of toxin/ml. Control cells were incubated with an equal volume of buffer.

RESULTS

Selective modulation of LTB₄ responsiveness by retinoic acid

HL60 cells exposed to retinoic acid or DMSO undergo morphological changes and acquire the ability to move, to generate superoxide anions and to ingest C3bi- or IgG-coated particles (Fontana *et al.*, 1980; Stendahl *et al.*, 1982; Kitagawa *et al.*, 1984). Approx. 60% of the cells become NBT-positive when stimulated with phorbol esters after 4 days of exposure to either DMSO or retinoic acid. However, a marked difference between DMSO- and retinoic acid-induced differentiation emerges when comparing $[Ca^{2+}]_i$ responses to two potent chemotactic agonists LTB₄ and fMet-Leu-Phe (Fig. 1).

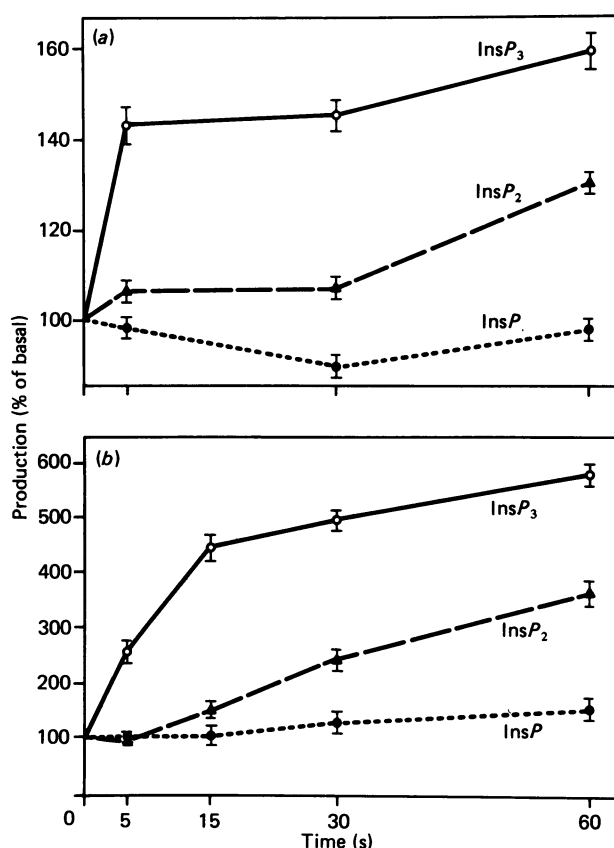


Fig. 2. Time course of formation of Ins P , Ins P_2 and Ins P_3 in LTB₄- and fMet-Leu-Phe-stimulated cells

(a) Shows the time course for the effect of LTB₄ (10^{-7} M) on Ins P , Ins P_2 and Ins P_3 production in retinoic acid-induced HL60 cells. (b) Shows the time course for the effect of fMet-Leu-Phe (10^{-6} M) on Ins P , Ins P_2 and Ins P_3 production in DMSO-induced HL60 cells. The reactions were stopped by addition of ice-cold trichloroacetic acid at the indicated times. Separation and extraction of lipids were performed as described in the Experimental section. The values are given as percentages of basal levels in control cells. Basal levels for retinoic acid-induced cells were 432 ± 18 , 98 ± 4 and 128 ± 10 d.p.m. for Ins P_3 , Ins P_2 and Ins P respectively and for DMSO-induced cells 306 ± 34 , 110 ± 13 and 128 ± 14 d.p.m. for Ins P_3 , Ins P_2 and Ins P respectively. The values represent the mean and S.D. of three determinations.

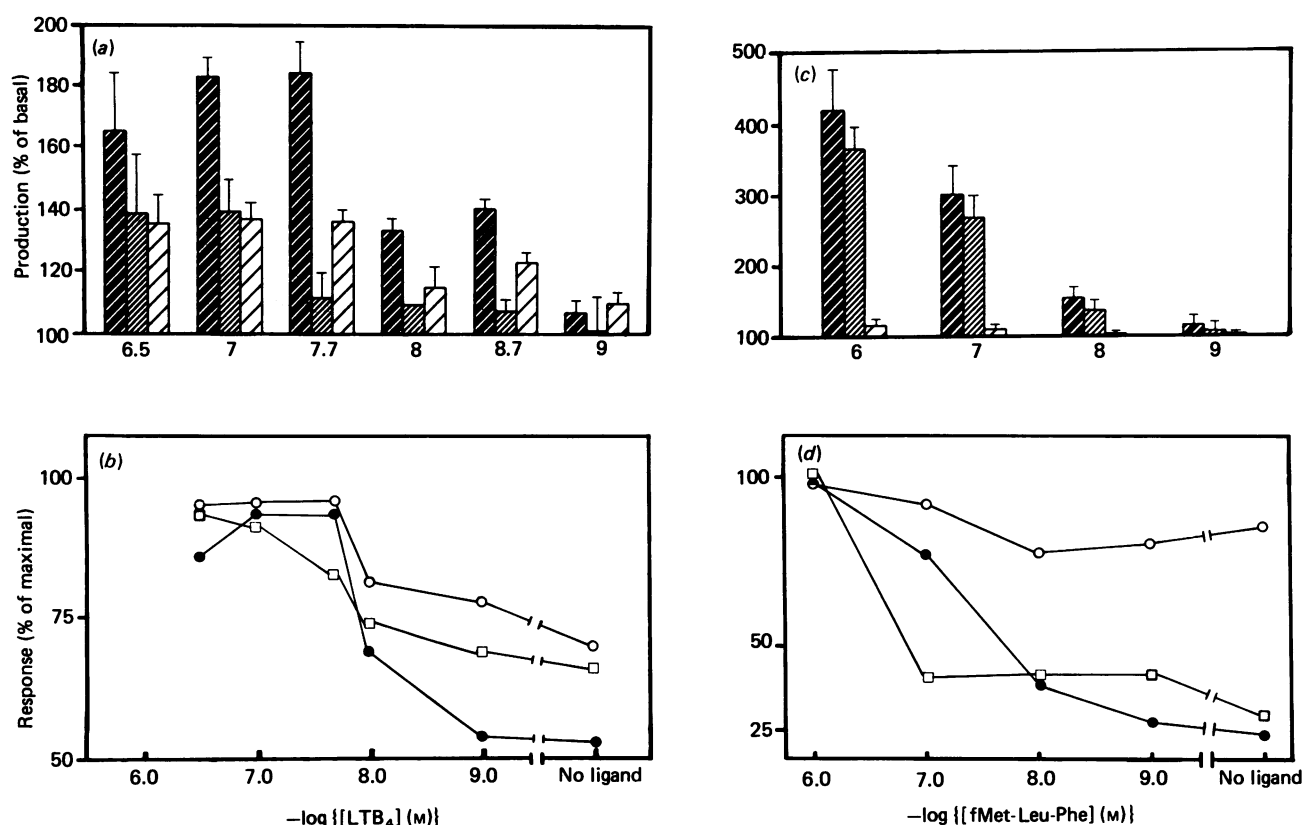


Fig. 3. Dose response for LTB₄- and fMet-Leu-Phe-induced formation of InsP₃, InsP₂ and InsP₃.

Shown are the dose-dependencies of InsP₃, InsP₂ and InsP production stimulated by LTB₄ and fMet-Leu-Phe in retinoic acid- and DMSO-differentiated HL60 cells respectively. In all cases the reactions were stopped 20 s after the addition of the stimulus by addition of ice-cold trichloroacetic acid, and inositol phosphates were determined as described in the Experimental section. In (a) and (c) the values are given as percentages of basal levels (mean \pm s.d. of three determinations). Basal levels for retinoic acid-induced cells were 230 ± 16 , 80 ± 6 and 103 ± 4 d.p.m. for InsP₃ (▨), InsP₂ (▤) and InsP (□) respectively and for DMSO-induced cells 211 ± 13 , 61 ± 13 and 56 ± 6 for InsP₃, InsP₂ and InsP respectively. In (b) and (d) the same data are redrawn expressed as a percentage of the maximal response in each experiment for LTB₄ and fMet-Leu-Phe; ●, InsP₃; □, InsP₂; ○, InsP.

Only retinoic acid-induced cells showed reproducible and quantitatively relevant responses to LTB₄. In Fig. 1(a) the maximal changes in $[Ca^{2+}]_i$ induced by LTB₄, expressed as changes in quin2 fluorescence, are shown. The $[Ca^{2+}]_i$ elevations were rapid and reached peak levels within a few seconds. Significant rises in $[Ca^{2+}]_i$, with a dose-response relationship similar to that observed for human neutrophils (Goldman *et al.*, 1985), could be elicited in retinoic acid-treated cells by LTB₄ at a threshold concentration ranging from 10^{-10} M to 10^{-9} M. In DMSO-induced cells the mean changes in $[Ca^{2+}]_i$ due to LTB₄ were weak, did not increase with increasing LTB₄ concentrations and were only observed in some batches of cells. As Fig. 1(b) shows, the $[Ca^{2+}]_i$ responses to fMet-Leu-Phe display an opposite pattern. Whereas HL60 cells exposed to DMSO respond similarly to neutrophils to low doses of fMet-Leu-Phe with a marked increase of $[Ca^{2+}]_i$, cells induced with retinoic acid require 100-fold higher doses of fMet-Leu-Phe to significantly raise $[Ca^{2+}]_i$. Under our experimental conditions HL60 cells can thus be selectively induced to acquire preferential responsiveness to either LTB₄ or fMet-Leu-Phe by retinoic acid and DMSO respectively.

Effects of LTB₄ on phosphoinositides and inositol phosphates

HL60 cells were exposed to inducers for 4 days and to myo-[³H]inositol during the last 48 h of the period of differentiation. This procedure leads to a steady state of labelling, indicating that the experiments are performed close to isotopic equilibrium. After LTB₄ stimulation of HL60 cells, differentiated with retinoic acid, there is a rapid increase in InsP₃ formation, a significant elevation being produced within 5 s after the addition of 10^{-7} M-LTB₄ (Fig. 2a). Increased InsP₃ levels are maintained during the first minute. There is also a rise in InsP₂; however, these changes are small and appear to lag behind the InsP₃ response. The effects of LTB₄ on InsP are variable, whereas there is no change in the experiment shown in Fig. 2(a), in some experiments InsP formation is modestly enhanced. For comparison with the effects of LTB₄ on inositol phosphate levels, the stimulation of DMSO-induced HL60 cells by fMet-Leu-Phe is shown in Fig. 2(b). There is a rapid rise in InsP₃ production similar to that stimulated by LTB₄, and after a lag of 5–15 s, InsP₂ formation gradually increases to a lesser amount.

However, a difference is observed when comparing the degree of maximal stimulation by the two agonists. Whereas fMet-Leu-Phe raises InsP_3 levels, measured 20 s after stimulation, to $257 \pm 22\%$ of control (mean \pm s.d., $n = 23$), LTB_4 only raises InsP_3 levels to $155 \pm 8\%$ of control (mean \pm s.d., $n = 13$).

Analysis of the phosphoinositide levels, measured 20 s after stimulation, reveals that neither LTB_4 nor fMet-Leu-Phe causes any significant changes (results not shown). Therefore enhanced phospholipase C hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ is compensated for by rapid resynthesis from PtdIns . The relative amounts of PtdIns , $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ are not significantly different in retinoic acid- as against DMSO-induced HL60 cells (results not shown). This observation makes it very unlikely that reduced substrate availability [$\text{PtdIns}(4,5)\text{P}_2$] in the retinoic acid-induced cells could be the cause of the difference between LTB_4 and fMet-Leu-Phe in their degree of maximal stimulation of InsP_3 production.

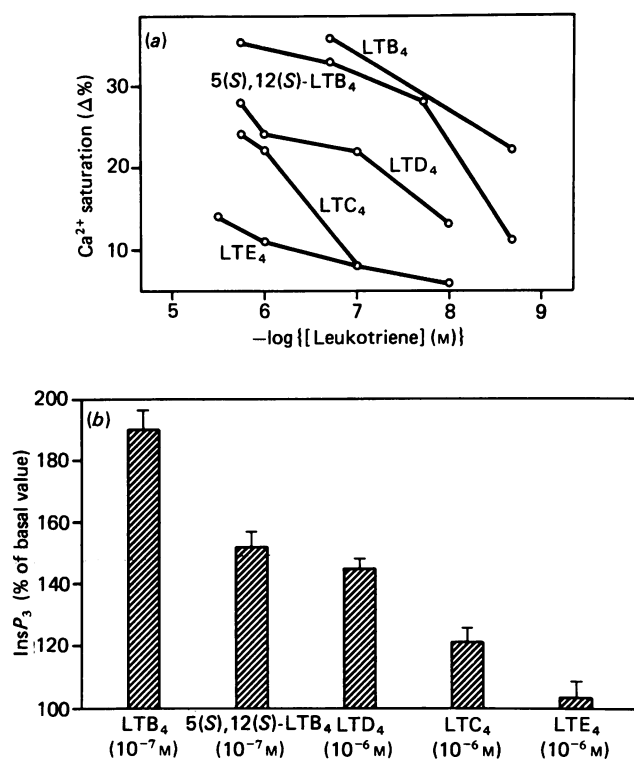


Fig. 4. Effects of leukotriene analogues on $[\text{Ca}^{2+}]_i$ changes and IP_3 formation

(a) Shows effects of various leukotrienes on maximal $[\text{Ca}^{2+}]_i$ rises in retinoic acid-differentiated HL60 cells. The leukotrienes used and concentrations are indicated in the Figure. (b) Shows effects of various leukotrienes on InsP_3 production. Measurements of Ca^{2+} transients and InsP_3 formation were carried out in the same batch of HL60 cells on the same day. The leukotrienes used and their concentrations are indicated in the Figure. All reactions were stopped by addition of ice-cold trichloroacetic acid 20 s after addition of stimulus. Separation and extraction of lipids were performed as described in the Experimental section. The stimulated InsP_3 productions are given as a percentage of basal levels in control cells. Basal levels for these cells were $119 \pm 13 \text{ d.p.m.}$ for InsP_3 . The values represents the mean and s.d. of three determinations.

Stimulation of InsP_3 production by LTB_4 is dose-dependent, with a threshold concentration of 10^{-9} M and a maximal stimulatory concentration around $5 \times 10^{-8} \text{ M}$ (Fig. 3a). In DMSO-treated cells exposed to fMet-Leu-Phe the threshold concentration is also around 10^{-9} M , and the maximal stimulatory concentration was found at 10^{-6} M (Fig. 3c). To allow simple comparison of agonist effects on the different inositol phosphate fractions, these results have also been calculated as percentage of maximal response (Figs. 3b and 3d). The specificity of the LTB_4 effect was evaluated with a stereochemical isomer of LTB_4 , $5(\text{S}), 12(\text{S})\text{-LTB}_4$, which also binds to the two subsets of LTB_4 receptors on human neutrophils (Samuelsson, 1983). This compound is capable of eliciting $[\text{Ca}^{2+}]_i$ rises, but higher concentrations are required to elicit maximal responses (Fig. 4a). The cysteine-containing leukotrienes, $\text{LTD}_4 > \text{LTC}_4 > \text{LTE}_4$, when ranking according to potency, are also capable of eliciting small, but statistically significant, rises in $[\text{Ca}^{2+}]_i$. However, even higher concentrations are required of these compounds. Our results show a very good correlation between the ability of these analogues to trigger InsP_3 generation and their capacity to elicit $[\text{Ca}^{2+}]_i$ changes (Fig. 4).

Effects of *Bordetella pertussis* toxin

The toxin has been shown to inhibit Ca^{2+} transients and functional responses elicited by LTB_4 and fMet-Leu-Phe in rabbit neutrophils (Becker *et al.*, 1985; Bradford & Rubin, 1985b). Therefore we investigated its effects on $[\text{Ca}^{2+}]_i$ levels and InsP_3 production in retinoic acid-induced HL60 cells. Preincubation of these cells with the toxin led to a total abolition of LTB_4 (10^{-7} M)-induced Ca^{2+} transients (not shown) and InsP_3 production ($98 \pm 4\%$ of the value for unstimulated cells not exposed to the toxin; mean \pm s.d., $n = 9$). Basal InsP_3 formation in toxin-treated cells also decreased slightly ($91 \pm 4\%$ of untreated control value; mean \pm s.d., $n = 6$). No change was observed in concentrations of the phospholipids PtdIns , $\text{PtdIns}4\text{P}$ or $\text{PtdIns}(4,5)\text{P}_2$, indicating that the decreased InsP_3 production in toxin-treated cells was not due to a decreased amount of substrate available for phospholipase C. However, the toxin did not affect the ability of neutrophils to respond to a stimulus that bypasses the plasma membrane, i.e. PMA (100 ng/ml). Both the initial rate of superoxide production and the number of cells capable of reducing NBT in response to PMA were identical in toxin-treated and in untreated control cells (results not shown).

Formation of the two isomers of InsP_3

Fig. 5 shows two typical elution profiles of LTB_4 -stimulated and fMet-Leu-Phe-stimulated HL60 cells respectively. Whereas $[\text{H}^3]\text{Ins}(1,3,4)\text{P}_3$ is eluted shortly after ATP, $[\text{H}^3]\text{Ins}(1,4,5)\text{P}_3$ follows approx. 1.8 min later. These elution profiles closely resemble those recently published (Burgess *et al.*, 1985; Irvine *et al.*, 1985). Fig. 6 shows the time course and relative proportion of formation between $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ in LTB_4 (Fig. 6a) as well as fMet-Leu-Phe-stimulated (Fig. 6b) HL60 cells. For both LTB_4 and fMet-Leu-Phe the Ca^{2+} -releasing isomer, $\text{Ins}(1,4,5)\text{P}_3$, exhibits a more rapid increase and a shorter duration when compared with the $\text{Ins}(1,3,4)\text{P}_3$ isomer. For both agonists the amount of the $\text{Ins}(1,4,5)\text{P}_3$ isomer was maximal at 5 s and declined to just above the starting

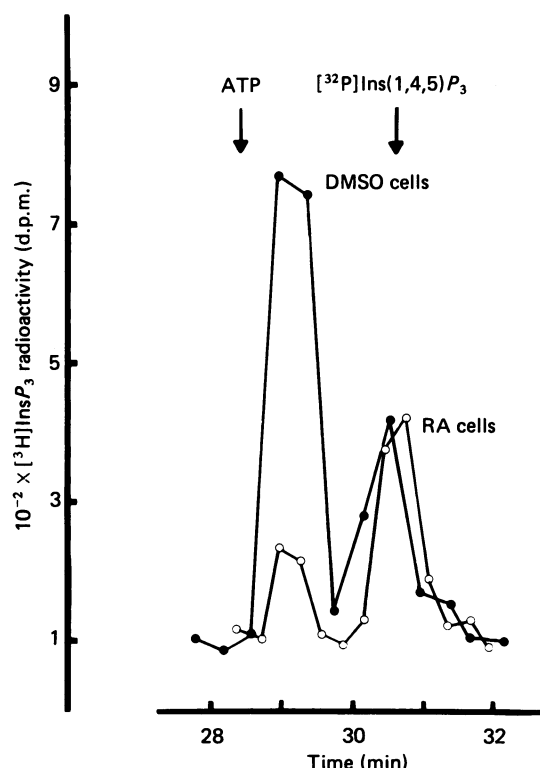


Fig. 5. Separation of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ by h.p.l.c.

Extracts of HL60 cells, differentiated with DMSO (DMSO cells) and stimulated with fMet-Leu-Phe ($1\ \mu\text{M}$) for 30 s (●), or differentiated with retinoic acid (RA cells) and stimulated with LTB_4 ($0.1\ \mu\text{M}$) for 5 s (○) were analysed by h.p.l.c. as described in the Experimental section. Arrows indicate the retention times for ATP and $[^{32}\text{P}]\text{Ins}(1,4,5)\text{P}_3$ respectively.

value after 1 min. The rapid elevations of $\text{Ins}(1,4,5)\text{P}_3$ parallel the $[\text{Ca}^{2+}]_i$ elevations in quin2-loaded HL60 cells (results not shown). In contrast, the amount of the $\text{Ins}(1,3,4)\text{P}_3$ isomer was maximal at 30 s and then only slowly declined with time. The kinetics of formation for the two isomers thus appear to be very similar for both LTB_4 and fMet-Leu-Phe (Fig. 6). Furthermore, a tight correlation was observed between the $\text{Ins}(1,4,5)\text{P}_3$ production and the $[\text{Ca}^{2+}]_i$ elevations elicited by various leukotriene analogues. The production of $\text{Ins}(1,4,5)\text{P}_3$ calculated from two separate experiments, in which all analogues were tested on the same batch of cells, were found to be: 236% for LTB_4 ($10^{-7}\ \text{M}$), 194% for $5(S),12(S)\text{-LTB}_4$ ($10^{-7}\ \text{M}$), 113% for LTC_4 ($10^{-6}\ \text{M}$) and 112% for LTD_4 ($10^{-6}\ \text{M}$) 5 s after stimulation (expressed as percentage of basal values).

DISCUSSION

Measurements of InsP_3 production in neutrophils presents several major methodological problems. Although these cells have the advantage of fully mature functional responses, they have a short life span. Therefore labelling with radioactive isotopes cannot be carried out for more than 18 h, a labelling time presumably too short for reaching isotopic equilibrium. Under these conditions neutrophils also lose some of their functional responsiveness (Di Virgilio *et al.*, 1985).

The promyelocytic cell line, HL60, can be induced to differentiate by DMSO or retinoic acid to express neutrophilic functions. These cells respond to the same low concentrations of LTB_4 as human neutrophils (Goldman *et al.*, 1985). In addition, differentiated HL60 cells have been shown to possess both cyclo-oxygenase and lipoxygenase functions similar to those of human neutrophils (Lundberg *et al.*, 1985). Cells maintained in culture, like HL60, have the advantage of allowing long-term labelling of phosphoinositides with *myo*- $[^3\text{H}]$ -inositol so that a steady state, presumably reflecting isotopic equilibrium, can be reached while the cells remain fully responsive. HL60 cells, therefore, provide an ideal experimental model for resolving the controversy about the implication of enhanced phospholipase C activity and increased formation of $\text{Ins}(1,4,5)\text{P}_3$ for LTB_4 stimulation of phagocytes.

The initial finding encountered in the present study was that, in HL60 cells induced by DMSO, a compound widely used for differentiation, stimulation with LTB_4 only led to weak $[\text{Ca}^{2+}]_i$ transients, and in some batches of cells the response was entirely absent. A fairly recent study (Benjamin *et al.*, 1985) demonstrated that LTB_4 binds equally well to HL60 cells regardless of whether they have been differentiated with DMSO (1.25% +

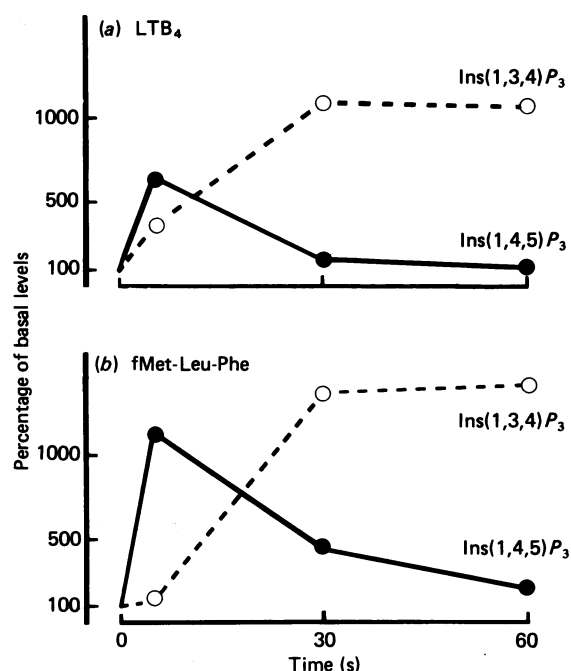


Fig. 6. Time course of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ formation

Retinoic acid- and DMSO-differentiated HL60 cells were stimulated with fMet-Leu-Phe ($1\ \mu\text{M}$) or LTB_4 ($0.1\ \mu\text{M}$) and analysed by h.p.l.c. for determination of their production of the two InsP_3 isomers. Results are expressed as a percentage of basal levels and indicate the mean result for two different experiments. Basal levels for retinoic acid-differentiated cells were 35 and 50 d.p.m. for $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ respectively and for DMSO-differentiated cells 40 and 60 d.p.m. for $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ respectively. Average increases in $\text{Ins}(1,4,5)\text{P}_3$ 5 s after introduction of LTB_4 or fMet-Leu-Phe were 420 ± 240 ($n = 4$) and 900 ± 300 ($n = 4$) respectively when calculated as a percentage of the basal value.

10 μ M-dexamethasone) or retinoic acid (1 μ M). The question as to why LTB₄, in DMSO-differentiated cells, fails to elicit a $[Ca^{2+}]_i$ transient similar to that in retinoic acid-differentiated cells remains open. In contrast, and of importance for the present study, after induction with retinoic acid (0.2 μ M for 4 days) there was a selective differentiation of cells that responded with very large $[Ca^{2+}]_i$ transients in response to LTB₄. Consequently, HL60 cells differentiated with retinoic acid are a powerful model to study the role of phosphoinositide metabolism and production of inositol phosphates for the LTB₄ stimulation of phagocytes.

The initial aim of our study was to solve the important controversy as to what events are involved in the LTB₄ transduction mechanism. Volpi *et al.* (1984) showed that fMet-Leu-Phe, but not LTB₄, at fully chemotactic concentrations, results in an increased breakdown of phosphatidylinositols in ³²P-labelled neutrophils. They also observed a small, but significant, increase of diacylglycerol in neutrophils stimulated with fMet-Leu-Phe but not in those stimulated with LTB₄. If these observations are correct, it would mean that LTB₄ is the first documented exception to the currently prevailing model in which Ins(1,4,5)P₃ is the main mediator of Ca²⁺ mobilization from intracellular stores (Berridge & Irvine, 1984). Theoretically LTB₄ could be capable of mobilizing intracellular Ca²⁺ by other mechanisms. For instance, LTB₄ has in the past been suggested to be a naturally occurring ionophore capable of transporting Ca²⁺ across lipid bilayers (Serhan *et al.*, 1982). In contrast, Bradford & Rubin (1985a), who used rabbit neutrophils actually labelled with *myo*-[³H]inositol, demonstrated that stimulation with 10⁻⁷ M-LTB₄, a concentration well beyond that necessary for a chemotactic response, resulted in a small, but significant, increase in InsP₃ formation. Our detailed study solves this controversy and directly demonstrates that LTB₄-receptor activation results in the formation of Ins(1,4,5)P₃, a second messenger for intracellular Ca²⁺ mobilization.

The parallel experiments with fMet-Leu-Phe performed in the present study are in good agreement with recent studies by Burgess *et al.* (1985) showing that this agonist elicits an increased production of total InsP₃ and Ins(1,4,5)P₃ in DMSO-induced HL60 cells. The rapid rise of Ins(1,4,5)P₃ for both agonists occurred at least in parallel with the increase of $[Ca^{2+}]_i$, supporting the idea that Ins(1,4,5)P₃ is the mediator of this rise. The InsP₃ production was dependent on the concentration of LTB₄, with threshold concentrations about 10⁻⁹ M, a 2–4-fold higher threshold concentration than that necessary to elicit $[Ca^{2+}]_i$ rises. This discrepancy between the presence of small $[Ca^{2+}]_i$ rises in the presence of low ligand concentrations and without detectable InsP₃ production has been discussed in detail by Michell *et al.* (1981) and Williamson *et al.* (1985). Those authors suggested that just a fraction of receptors must be occupied to generate maximal $[Ca^{2+}]_i$ rises. With higher receptor occupancy, there is an overproduction of InsP₃ beyond that required for Ca²⁺ mobilization. Although no mass determination of InsP₃ production has been carried out in the present study, we observe a more impressive production of InsP₃ and Ins(1,4,5)P₃ (compared with basal levels) in response to fMet-Leu-Phe than the response to LTB₄. Such a difference in inositol metabolism for the two agonists is not, as discussed above, contradictory to our finding that both agonists

are capable of raising $[Ca^{2+}]_i$ to similar peak levels. However, signals other than a rise of the cytosolic free Ca²⁺ level, such as diacylglycerol production and protein kinase C activation, might well be influenced and explain the different biological effects of LTB₄ and fMet-Leu-Phe.

The specificity of the LTB₄ effect was demonstrated by the strong correlation between abilities of the various leukotrienes to raise $[Ca^{2+}]_i$ and to stimulate both total InsP₃ and Ins(1,4,5)P₃ production. The smaller effects elicited by the thiol-containing leukotrienes are presently unexplained and require further investigation to assess whether they bind to LTB₄ receptors or other types of receptors. Similar levels of phospholipid precursors, in particular PtdIns(4,5)P₂, indicate that the smaller amount of InsP₃ produced (compared with basal values) in response to LTB₄ was not due to a decreased concentration of phospholipid precursor. Long-term labelling of phospholipids with *myo*-[³H]inositol allows assessment of the relative quantity of PtdIns, PtdIns4P and PtdIns(4,5)P₂ alterations, whereas short-term labelling with [³²P]phosphate only indicates fluctuation in radioactive label. In the present study we observed no significant alteration in phosphoinositide levels after stimulation with either LTB₄ or fMet-Leu-Phe. This indicates that rapid resynthesis of phosphoinositides compensates for the loss by phospholipase C activity. In light of these results, it is evident that, in the ³²P-labelling experiments previously described (Volpi *et al.*, 1984), only transient changes of phospholipase C activity due to a strong stimulus like fMet-Leu-Phe, but not due to a weaker stimulus like LTB₄, could be observed.

The inhibitory effect of *Bordetella pertussis* toxin on $[Ca^{2+}]_i$ transients, reflecting both intracellular mobilization and influx of Ca²⁺, and InsP₃ production elicited by LTB₄ provide further information on the nature of the coupling system that links LTB₄ to phospholipase C. The toxin ADP-ribosylates a G (or N) regulatory coupling unit and by this action inhibits the function of these proteins (Rodbell, 1980; Gilman, 1984). In human neutrophils and differentiated HL60 cells it has been shown that the toxin inhibits fMet-Leu-Phe-receptor-induced intracellular signals, in particular InsP₃ production and functions (Krause *et al.*, 1985; Becker *et al.*, 1985; Bradford & Rubin, 1985b; Brand *et al.*, 1985), which strongly suggests that a coupling G protein links the receptor to phospholipase C. Furthermore, the observations that pertussis toxin entirely inhibits both the fMet-Leu-Phe (Krause *et al.*, 1985) and the LTB₄-induced $[Ca^{2+}]_i$ rise as well as the increased production of InsP₃ makes it tempting to speculate that products of polyphosphoinositide breakdown, apart from mobilizing Ca²⁺ from intracellular stores, might also be involved in the regulation of Ca²⁺ influx.

The present data thus indicate that LTB₄ receptors have similar characteristics to fMet-Leu-Phe receptors, in regard to their dependency on a G regulatory coupling unit and to the fact that they generate Ins(1,4,5)P₃, a second messenger for intracellular Ca²⁺ mobilization. Furthermore, the initial studies of [³H]LTB₄ binding, receptor signal transduction and function in myeloid differentiated HL60 cells by us and others suggest that this cell line will prove a powerful tool in the study of the mode of action of LTB₄.

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